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Article

Increased Levels of Hydrogen Peroxide Induce a HIF-1-dependent Modification of Lipid Metabolism in AMPK Compromised *C. elegans* Dauer Larvae

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SUMMARY

Cells have evolved numerous mechanisms to circumvent stresses caused by the environment, and many of them are regulated by the AMP-activated kinase (AMPK). Unlike most organisms, *C. elegans* AMPK-null mutants are viable, but they die prematurely in the "long-lived" dauer stage due to exhaustion of triglyceride stores. Using a genome-wide RNAi approach, we demonstrate that the disruption of genes that increase hydrogen peroxide levels enhance the survival of AMPK mutant dauers by altering both the abundance and the nature of the fatty-acid content in the animal by increasing the HIF-1-dependent expression of several key enzymes involved in fatty-acid biosynthesis. Our data provide a mechanistic foundation to explain how an optimal level of an often vilified ROS-generating compound such as hydrogen peroxide can provide cellular benefit, a phenomenon described as hormesis, by instructing cells to readjust their lipid biosynthetic capacity through downstream HIF-1 activation to correct cellular energy deficiencies.

INTRODUCTION

Throughout the course of evolutionary history, environmental fluctuations have played a major role in determining both the number and the morphological diversity of our current biota. Factors such as limiting resource availability and/or accessibility were most likely instrumental in driving adaptations that, among other effects, were capable of enhancing survival during periods of environmental duress by impinging upon behavior and metabolic changes that improved survivability. This is well demonstrated in animals that have adapted to prolonged periods of nutrient deprivation. In the case of bears, for example, hibernation is preceded by a highly active preparatory phase during which the animal increases its body weight by 40%, mostly in the form of both brown and white adipose tissue (Jonkel and Cowan, 1977). In rodents and humans, extensive physical stress/activity or nutrient stress is often accompanied by hormonal imbalance and reproductive arrest, presumably to ensure that development is paused when macromolecular components are limiting. In doing so, animals link physiology to both development and behavior (Holliday, 1989; Kirkwood, 1977; Selesniemi et al., 2008).

This is not unique to mammals, as many invertebrates have adapted diapause-like states to enhance survival during severe environmental stresses. Many parasitic nematodes develop through a highly resistant "dauer" stage that is important to escape host defenses during infection (Riddle and Georgi, 1990), while in the free-living nematode *C. elegans*, the dauer stage is not obligatory, but instead provides the animal with an effective means of dispersal and increased stress resistance.

In *C. elegans*, three parallel genetic pathways converge to ensure cell-division arrest, behavioral and morphological alterations, and, finally, a phase of global metabolic change that are all characteristic of dauer formation. One common downstream effector of these three pathways is the AMP-activated protein kinase (AMPK) (Narbonne and Roy, 2006). After activating phosphorylation by LKB1/PAR-4, it will phosphorylate targets that block anabolic processes and activate gene products involved in energy generation (Hawley et al., 2003; Woods et al., 2003; Hardie, 2007).

In AMPK mutant dauer larvae, the germline contains greater than 4-fold the normal complement of germ cells (Narbonne and Roy, 2006). Independent of any effects on the germline, the larvae die after 10 days because, despite their transient accumulation of fat upon dauer entry, they hydrolyze it rapidly and deplete their fat reservoirs in less than 48 hr after dauer formation (Narbonne and Roy, 2009).

In most animals, fat is stored in the form of triglycerides, and the hydrolysis of these triglycerides into free fatty acids is catalyzed by adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004). In *C. elegans*, ATGL-1 is phosphorylated and inhibited by AMPK to preserve the triglyceride stores for use during the course of the diapause (Narbonne and Roy, 2009). Loss of AMPK in *C. elegans* dauer larvae leads to the misregulation of ATGL-1, resulting in rapid mobilization of the triglyceride stores, and the consequent premature expiration of the larvae (Narbonne and Roy, 2009).

Removal of ATGL-1 partially rescues the premature lethality of AMPK mutant dauer larvae; therefore, we reasoned that we could identify factors that act downstream of AMPK to enhance ATGL-1 function, to preserve the lipid stores and prolong survival of the AMPK mutant dauer larvae. Using an RNA interference (RNAi) feeding strategy, we identified 551

genes that, when compromised, enhanced the survival of AMPK mutant dauer larvae. Surprisingly, the loss of any one of the three *C. elegans* catalase (*ctl*) genes could suppress all the previously described metabolic defects associated with compromised AMPK function.

Our characterization of the effects of catalase mutations on AMPK mutant dauer survival reveal a hitherto unforeseen role of H_2O_2 in activating HIF-1 in vivo, which alters cell physiology to favor growth and survival under stress. Moreover, we provide molecular evidence to explain H_2O_2/ROS-mediated hormesis, while underscoring the need to establish a fine equilibrium between the beneficial and harmful effects of these oxygen species.

RESULTS

A Global Genome Survey to Identify Genes that Enhance the Survival of AMPK Mutant Dauer Larvae

Loss of AMPK in *C. elegans* dauer larvae results in premature arrest due to exhaustion of fat reserves that would normally sustain the animal throughout the dauer stage (Narbonne and Roy, 2006). Compromise of ATGL-1 partially rescued the dauer-dependent lethality and the abnormal loss of lipid in AMPK compromised dauer larvae. To identify genes that cooperate with ATGL-1 and/or that may act downstream of AMPK in energy regulation, we conducted a global genome survey using an RNAi feeding strategy (Kamath et al., 2001). We screened over 18,000 genes for candidates that, when compromised, enhanced the survival of AMPK mutant dauer larvae. The *atgl-1* RNAi clone was identified randomly as a positive candidate from our primary screen, assuring us that our strategy was specific and effective. Five hundred and fifty-one additional RNAi clones were identified, for which about 60% have been assigned a functional signature based on gene ontology and/or available experimental data (Figure 1A).

We noted that the compromise of any one of two catalase genes in the library (*ctl-1* and *ctl-2*) increased the survival of AMPK mutant dauers (Figure 1B and Table S2 available online). Catalases play an important role in the neutralization of hydrogen peroxide (H_2O_2) and thus may regulate the cellular load of reactive oxygen species (ROS). Because ROS impinges on so many cellular processes including life span and lipid metabolism, we chose to further study how the loss of catalase function might affect these processes and hence enhance dauer survival in AMPK mutants.

We obtained three mutant strains, each of which has a single deletion in each of the catalase family members: *ctl-1* (*ok1242*), *ctl-2* (*ok1137*), and *ctl-3* (*ok2042*). The *ctl-1* mutation is a severe loss of function allele (Coolon et al., 2009), while *ctl-2* (*ok1137*) and *ctl-3* (*ok2042*) are unlikely to encode functional proteins (Boon et al., 2007).

AMPK mutant dauer larvae expire prematurely due to rapid mobilization of their triglyceride stores and failure of the excretory system to maintain osmotic balance (Narbonne and Roy, 2009). Surprisingly, mutation of any one of the *ctl* genes significantly increased the survival of AMPK mutant dauers, most notably when *ctl-3* function was compromised (Figure 1C and Table S2). In addition, each of the three alleles restored total lipid and triglyceride levels to near wild-type levels (Figures 1D and 1E) and corrected the osmoresistance defect, especially at high salt concentrations (Figure 1F). Conversely, the germline hyperplasia typical of AMPK mutant dauers was unaffected by any of the catalase mutations.

Curiously, the catalase mutations do not provide the same survival benefit in AMPK mutant dauers that are induced with pheromone (data not shown). The reason for this distinction is not yet clear, but it is reminiscent of the differential effects of the dauer-inducing mutations on life-span extension, wherein only *daf-2* mutations exhibit any appreciable effect (Kimura et al., 1997). This unique aspect of the *daf-2* mutants probably reflects the metabolic readjustment that these larvae undergo in response to the nutrient stress that accompanies a block in insulin signaling, which does not occur in dauer larvae where the insulin-like signaling pathway remains active.

Taking these data together, we conclude that compromise of any individual catalase gene function is sufficient to restore the regulated breakdown of stored triglycerides over a prolonged duration, while consequently re-establishing excretory/osmoregulatory function in the AMPK mutant dauer larvae.

Disruption of Catalase Function Increases Lipid Droplet Size and Attenuates ATGL-1 Activity in AMPK Mutant Dauers

Since we observed a significant improvement in lipid retention in AMPK mutant dauers that lacked catalase, we questioned whether the compromise of *ctl* function could differentially affect ATGL-1 activity and thus account for the accumulation and maintenance of fat stores. To meet this end, we measured the total triglyceride lipase activity in extracts obtained from both AMPK and catalase-deficient AMPK mutant dauer larvae, and we found that it was decreased by two-fold in the *ctl*-compromised AMPK mutant dauers (Figure 2A). We performed *atgl-1(RNAi)* on the catalase-deficient AMPK mutant dauers to determine whether the reduction in lipase activity could be attributed to effects on ATGL-1. The attenuated lipase activity in these catalase-deficient mutant dauers was not further affected by *atgl-1(RNAi)*, suggesting that the loss of catalase function ultimately blocked ATGL-1 activity in these double-mutant dauers (Figure 2A).

In mammalian cells, ATGL is under complex regulation by CGI-58 and Perilipin, both of which reside on lipid droplets in the adipose tissue (Schweiger et al., 2008; Gruber et al., 2010). Recent data showed that the disruption of peroxisomal β-oxidation expands the size of lipid droplets and was accompanied by an increase in triglyceride levels (Zhang et al., 2010). Because changes to the lipid droplet composition or architecture could contribute to the net activity of ATGL, we wondered whether the loss of *ctl* function affects the capacity of ATGL-1 to hydrolyze the lipid reserves in AMPK mutant dauer larvae by altering the morphology of the lipid droplets. By fluorescently staining lipids and with oil red O, we noted a significant expansion in lipid droplet size in day 1 *ctl*-deficient AMPK mutant dauers (Figures 2G–2K and 2C–2F), which was most pronounced in *ctl-3*-deficient AMPK mutant dauers. The size expansion of the lipid droplets observed in catalase mutant dauers could be beneficial at two levels: first, they could provide additional storage volume to accommodate an increased level of triglycerides in catalase-deficient AMPK mutant dauers (Figures 1D and 1E), and second,

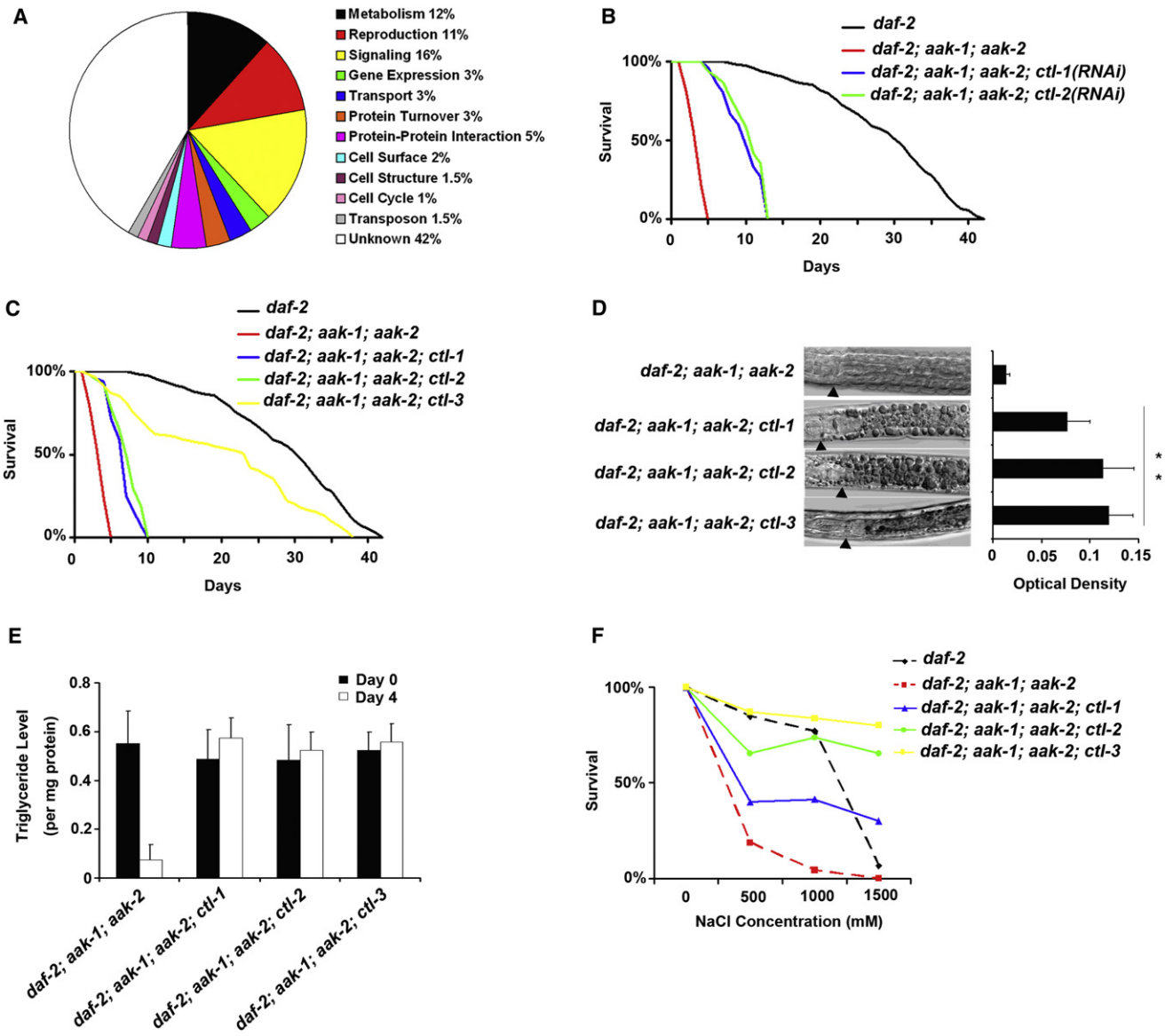

Figure 1. A Genome-wide RNAi Survey for Suppressors of AMPK-Dependent Dauer Lethality

(A) After analysis of 18,000 predicted genes included in the RNAi-feeding library, 551 RNAi clones were identified that extend the survival of *aak-1; aak-2* [*aak(0)*] dauer larvae that die after 10–12 days in culture. Candidate genes were assigned gene ontology terms based on information provided on WormBase (http://www.wormbase.org/). Details of the individual genes are listed in Table S1.

(B) Both *ctl-1* and *ctl-2* RNAi clones were able to significantly increase the survival of *aak(0)* mutant dauer larvae. Dauer larvae were maintained at 25°C as described elsewhere (Narbonne and Roy, 2006). Raw dauer survival values are shown in Table S2.

(C) Each of the *ctl-1(ok1242)*, *ctl-2(ok1137)*, and *ctl-3(ok2042)* mutations were introduced into the *daf-2(e1370); aak-1(tm1944); aak-2(ok524)* background, and their individual effects on survival, triglyceride content and osmosensitivity were assessed as in Narbonne and Roy (2009).

(D) Mutation of any of the three *ctl* genes protects triglyceride stores from depletion in day 4 AMPK mutant dauers. Fat storage was visualized by oil red O staining of day 4 dauer larvae. Arrowheads indicate the junction between the pharynx (left) and the intestine (right). Oil red O staining intensity was evaluated by measurement of optical density. Error bars indicate the SD of 20 animals. * and ** indicate statistical significance ($p < 0.05$ and $p < 0.01$, respectively) compared to AMPK mutant dauer larvae with one-way ANOVA followed by a Tukey HSD test. The same statistical analysis was applied for all additional experiments performed hereafter.

(E) Colorimetric analysis of triglyceride content in day 0 and 4 dauer larvae. Error bars indicate the SD of three independent experiments.

(F) Mutation of any one of three *ctl* genes enhances the osmoresistance of day 4 AMPK mutant dauers after maintenance in varying NaCl concentrations for 24 hr at 25°C.

See also Tables S1 and S2.

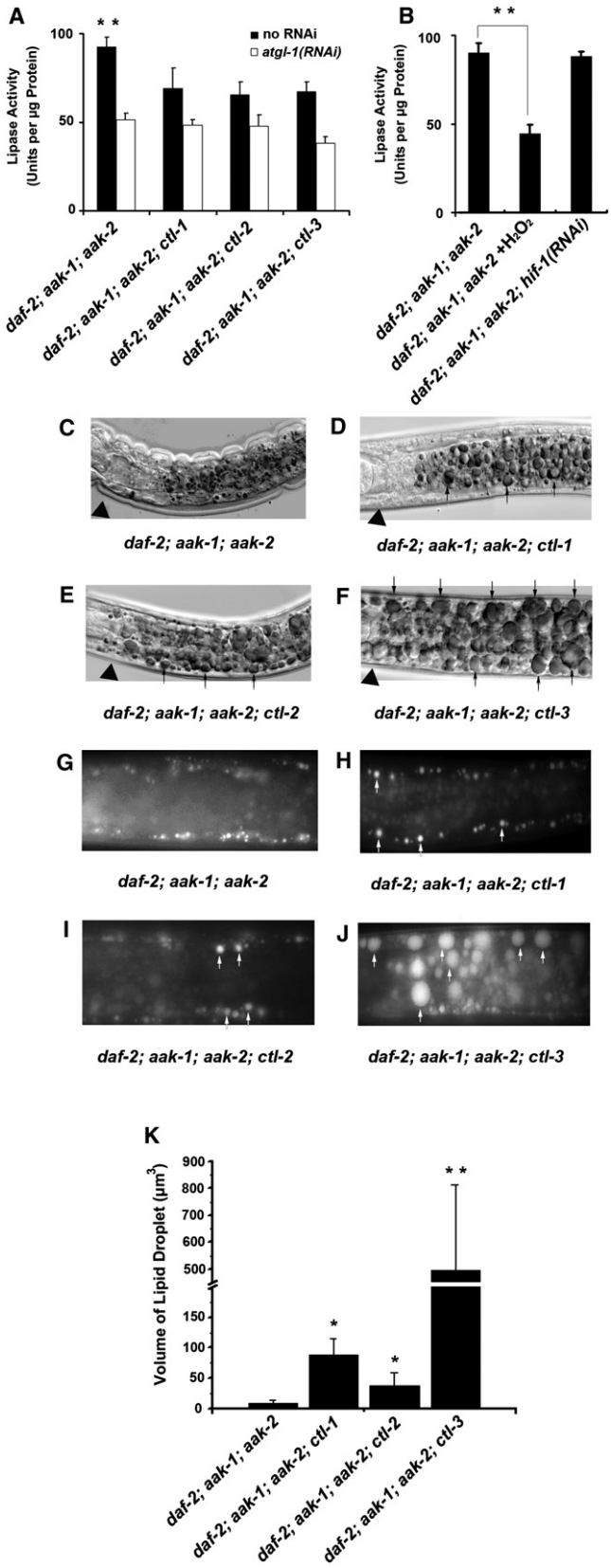

Figure 2. Disruption of Catalase Genes Results in the Attenuation of ATGL-1 Activity and Expansion of Lipid Droplet Size in AMPK Mutant Dauers

(A) Total lipase activity was significantly reduced in AMPK *aak(0); ctl* mutant dauers, while *atgl-1(RNAi)* feeding was 2-fold more efficient in reducing the overall lipase activity of AMPK mutant dauer larvae. *aak(0); ctl; atgl-1(RNAi)* combinations reduced lipase activity to levels typical of *atgl-1(RNAi)* alone. Error bars indicate the SD of three independent experiments.

(B) Total lipase activity was significantly reduced in H_2O_2-treated AMPK *aak(0)* mutant dauers but not those subjected to *hif-1(RNAi)*. Error bars indicate the SD of three independent experiments.

(C–F) Oil red O staining of day 1 dauer larvae reveals an expansion in lipid droplets (arrows) in AMPK *aak(0); ctl* mutant dauers. Arrowheads indicate the junction between the pharynx (left) and the intestine (right).

(G–J) BODIPY-labeled day 1 dauer larvae delineates size expansion in lipid droplets (arrows) in AMPK *aak(0); ctl* mutant dauers.

(K) Volume quantification of the BODIPY-stained lipid droplet structures was performed as described elsewhere (Zhang et al., 2010) with OpenLab software (Improvision, UK). Error bars indicate the SD of 20 animals.

the enlarged lipid droplets could alter the enzymatic activity of ATGL-1 and thereby affect its rate of triglyceride hydrolysis.

H_2O_2 Produced from Fatty-Acid β-Oxidation in the Peroxisomes Increased the Dauer Survival of AMPK CTL Mutant Dauers

Since the major function of catalase is to convert H_2O_2 into water and oxygen, we questioned whether the accumulation of H_2O_2 caused by the loss of catalase function was responsible for the enhanced survival of AMPK mutant dauers. We therefore assessed the levels of both total ROS and H_2O_2 in catalase-deficient AMPK mutant dauers and compared the levels with AMPK mutant dauer larvae. The total ROS levels were significantly decreased in AMPK single mutants compared to control *daf-2* mutant dauer larvae, while mutation of any individual *ctl* gene marginally increased ROS levels in AMPK mutant dauers (Figure 3A). In contrast, the H_2O_2 levels were markedly increased in all catalase-deficient AMPK mutant dauers, which could be reversed by treatment with the potent antioxidant N-acetylcysteine (NAC) (Figure 3B). This suggests that the loss of catalase contributes more significantly to the overall levels of H_2O_2 rather than other ROS species that are present in these mutant dauers. To further confirm the effect of increased H_2O_2 levels on dauer survival, we treated the catalase-deficient AMPK mutant dauers with 10mM NAC, and, in parallel, we cultured AMPK mutant dauers in the presence of H_2O_2 or with paraquat (Pq), a strong ROS-generating compound, to determine their effects on dauer survival. Consistent with the idea that the increase in H_2O_2 levels confers the beneficial effect on dauer survival, treatment with 0.1% H_2O_2, but not 10 µM paraquat increased the survival of AMPK mutant dauers (Figures 3F and 3G and Table S2). Moreover, treatment with 10 mM NAC reversed the effect associated with catalase disruption and reduced the survival of all three strains of catalase-deficient AMPK mutant dauers (Figures 3C–3E and Table S2). These results suggest that it is the increased level of H_2O_2 that consequently led to the prolonged survival of catalase-deficient AMPK mutant dauers.

One major source of H_2O_2 production arises from fatty-acid oxidation that occurs in the peroxisome. Since the increased survival of catalase-deficient AMPK mutant dauers was related to changes in the mobilization of the triglyceride stockpile, we

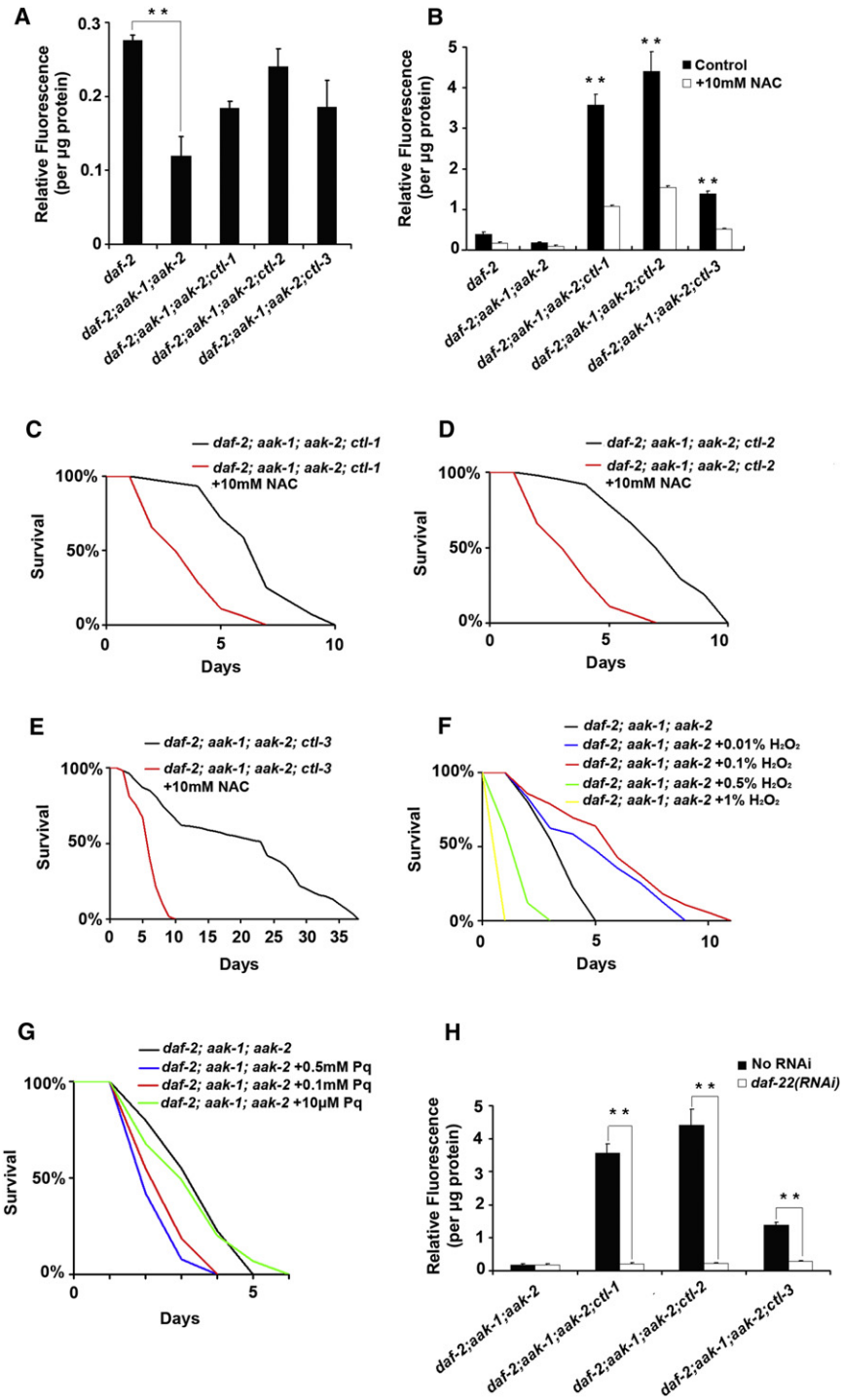

Figure 3. Elevated Concentrations of H_2O_2 Enhance the Survival of AMPK Mutant Dauers

(A) Less ROS was produced in AMPK mutant dauers (*daf-2*) as assessed with the total ROS dye indicator DCF-DA, while mutation of individual *ctl* genes only partially restored ROS levels. Error bars indicate the SD of three independent experiments.

(B) Total H_2O_2 levels were significantly increased in AMPK *aak(0); ctl* mutant dauers, which could be reversed by treatment with 10 mM NAC. Error bars indicate the SD of three independent experiments.

(C–E) The *ctl*-dependent enhancement of *aak(0)* dauer survival is reversed by antioxidant treatment with 10 mM NAC.

(F) Treatment with 0.1% and 0.01% H_2O_2 prolonged AMPK mutant dauer survival but higher concentrations of H_2O_2 were toxic. Assays were carried out under light-sensitive conditions to minimize H_2O_2 breakdown during assays.

(G) Paraquat (Pq; 10 µM) has no effect on the survival of *aak(0)* dauers. Lower concentrations from 1–10 µM did not affect dauer survival (data not shown), while higher concentrations (0.1 mM and 0.5 mM) were toxic and reduced dauer survival.

(H) The increased hydrogen peroxide levels typical of *aak(0); ctl* mutant dauers are reversed after the disruption of peroxisomal fatty-acid oxidation by *daf-22(RNAi)*. Error bars indicate the SD of three independent experiments.

See also Figures S1, S3, S5, and S6 and Table S2.

questioned whether the elevated levels of H_2O_2 observed in the catalase-deficient dauer larvae may be produced from peroxisomal fatty-acid β-oxidation. To verify this, we eliminated *daf-22* gene function, which disrupts the final step in peroxisomal fatty-acid β-oxidation, in both AMPK and catalase-deficient AMPK mutant dauer larvae to determine whether the increased H_2O_2 production could be reversed in the latter. Disruption of peroxisomal fatty-acid β-oxidation was sufficient to reverse the observed elevated H_2O_2 levels in the catalase-deficient AMPK mutant dauers (Figure 3H). We further noted that the compromise of several mitochondrial genes could also extend dauer survival, potentially by increasing flux through the peroxisomal β-oxidation pathway and generating increased H_2O_2 byproducts (Figure S3 and Table S2). These candidates were not identified in our initial screen because the affected animals expire before reaching the cutoff we established at 10 days after dauer. Since H_2O_2 is a major product of fat catabolism, the accumulation of the reaction products could potentially affect ATGL-1 activity via product inhibition (Walter and Frieden, 1963).

Taken together, our results suggest that increased peroxisomal fatty-acid β-oxidation results in elevated H_2O_2 levels that accumulate in catalase-deficient AMPK mutant dauers. This increase in H_2O_2, might account for the attenuated lipase activity, presumably by affecting ATGL-1, or alternatively through protecting the accumulated fat stores in the larva from rapid depletion.

Increased Survival of AMPK CTL Mutant Dauers Is HIF-1 Dependent

Given that modest increases in H_2O_2 or ROS can stimulate the hypoxia-inducible transcription factor HIF-1 to activate gene expression and promote longevity (Chandel et al., 1998; Lee et al., 2010), we determined whether the increased H_2O_2 generated in catalase-deficient AMPK mutant dauers could induce HIF-1 activation to ultimately prolong survival. In order to monitor the activation of HIF-1, we introduced a HIF-1-responsive GFP reporter, *Pnhr-57::GFP*, into the catalase-deficient AMPK mutant and AMPK mutant dauer larvae and monitored GFP expression during the dauer stage (Miyabayashi et al., 1999). We observed an increase in *Pnhr-57::GFP* expression in all three of the catalase-deficient AMPK mutant dauer backgrounds (Figure 4A). To verify that the increased expression of the HIF sensor indeed resulted from the increased H_2O_2 levels in the various mutants, we monitored GFP reporter expression in AMPK mutant dauers harboring the HIF-1 sensor after treatment with 0.1% H_2O_2. Indeed, the level of *Pnhr-57::GFP* was also significantly increased compared to untreated AMPK mutant dauers (Figures 4A and 4B).

HIF-1 activity is regulated predominantly by affecting its stability, which in turn is controlled by the prolyl hydoxylase (PHD)-mediated modification of the HIF-1 protein (Epstein et al., 2001; Cockman et al., 2000). Optimal PHD activity is highly dependent on O_2 levels and its cofactors, Fe II and 2-oxoglutarate (Epstein et al., 2001). Previous findings indicated that H_2O_2 may oxidize Fe II to Fe III, thereby inhibiting PHD to ultimately stabilize HIF-1 (Gerald et al., 2004). If the increased amount of H_2O_2 observed in the catalase-deficient AMPK mutant dauer larvae affects the cellular oxidation state of Fe II, this change could result in HIF-1 stabilization despite otherwise normoxic conditions. Our data are consistent with this since HIF-1 accumulates in the *ctl-1*, *ctl-2*, and *ctl-3* mutant backgrounds to levels that are near the range of those seen with *vhl-1(RNAi)*, while similar HIF-1 stabilization is observed by treatment of animals with 0.1% H_2O_2 (Figure 4C). Somewhat unexpectedly, we did not note any effect of AMPK depletion on HIF-1 levels, contrary to findings reported in other contexts (Shackelford et al., 2009; Shaw, 2006; Brugarolas and Kaelin, 2004). We further assessed HIF-1 protein levels in catalase-deficient AMPK mutant dauers treated with 10 mM NAC, which resulted in a significant drop in HIF-1 levels (Figure 4D) that was reversed after a combined treatment of H_2O_2 and NAC (Figure S4). No effect was observed after a combined treatment of NAC and *vhl-1(RNAi)* (Figure S4). We interpret these data to indicate that the increased H_2O_2 generated in catalase-deficient AMPK mutant dauer larvae stabilizes HIF-1, potentially by blocking PHD function.

To determine whether the activation of HIF-1 was associated with the prolonged survival of the catalase-deficient AMPK mutant dauers, we eliminated HIF-1 activity in these dauers and assessed its effects on dauer survival. *hif-1(RNAi)* reduced dauer survival in all three catalase-deficient AMPK mutant strains (Figures 4E–4G and Table S2). Conversely, *vhl-1(RNAi)* and *egl-9(RNAi)* both extended the survival of AMPK mutant dauers (Figure 4H and Table S2). These results suggested that the elevated H_2O_2 levels in the catalase-deficient AMPK mutant dauers activate HIF-1-dependent transcription, which consequently prolongs the survival of the AMPK mutant dauers.

HIF-1 Activates Expression of Genes Involved in Fatty-Acid Biosynthesis

Because the reduction of catalase activity was sufficient to restore lipid levels to near wild-type levels in AMPK mutant dauers, we surmised that HIF-1 activation would have to impinge on lipid biosynthesis. We therefore analyzed the fatty-acid composition of AMPK and catalase-deficient AMPK mutant dauers using gas chromatography/mass spectrometry (GC/MS), and we noted that additional monomethyl branched-chain fatty-acid species (C15ISO and C17ISO) and significantly more polyunsaturated long-chain fatty acids (PUFAs) were present in the AMPK mutant dauers that lacked catalase when compared to *daf-2: aak(0)* dauers (Figure 5A). The branched fatty acids originate almost entirely from the de novo synthesis pathway (Perez and Van Gilst, 2008), which may be stimulated in the dauer larvae that lack catalase. However, because of the inherent limitations in our method of analysis, we were unable to quantitatively assess the rate of de novo fatty-acid synthesis. Nevertheless, the loss of catalase function caused a substantial increase in triglyceride levels in these dauer animals, despite the lack of dietary intake, while also affecting the accumulation of branched chain fatty acids and PUFAs.

The rate of fatty-acid synthesis is under strict control by enzymes that catalyze several rate-limiting reactions and HIF-1 could affect lipid biosynthesis by increasing the expression of key enzymes involved in any one or several of these steps. To address this possibility, we analyzed the expression of enzymes involved in fatty-acid synthesis and noted that the expression of *fat-2*, *fat-3*, *fat-5*, *fat-6*, *fat-7*, *elo-5*, and *elo-6* were all significantly increased in AMPK mutant dauers that lacked catalase, especially in those that lacked *ctl-3* (Figure 5B). FAT-2 is involved in the desaturation of oleate (C18:1) and FAT-3 desaturates linoleate (C18:2) and alpha-linolenate (C18:3n-3), respectively, while FAT-5 catalyzes the desaturation of palmitate (C16:0) to palmitoleate (C16:1). The FAT-6 and FAT-7 desaturases mainly affect the generation of oleate (C18:1n9) using stearate (C18:0) as a substrate, while ELO-5 and ELO-6 catalyze the formation of C15ISO and C17ISO (reviewed by Watts, 2009). All of these enzymes catalyze rate-limiting steps in the synthesis of fatty acids in *C. elegans*, the transcript levels of which are all greatly affected by the loss of catalase activity in AMPK mutant dauers. Based on these observations, we suggest that after its activation in the animals lacking catalase, HIF-1 promotes the survival of AMPK mutant dauers by activating the transcription of enzymes involved in the rate-limiting desaturase and elongation steps during fatty-acid synthesis. To test this further, we performed *hif-1(RNAi)* in catalase-deficient AMPK mutant dauers and assessed their fatty-acid composition using GC/MS. Through elimination of HIF-1, the additional fatty-acid species observed in the catalase-deficient AMPK mutant dauers disappeared and the overall fatty-acid profile resembled that of AMPK mutant dauers (Figures 5C–5E). To further confirm the role of HIF-1 and H_2O_2 in affecting fatty-acid biosynthesis, we verified whether both *hif-1(RNAi)* and 10 mM NAC treatment could also reverse the observed increases in expression of the rate-limiting enzymes required for fatty-acid biosynthesis. Consistent with a role for HIF-1 in activating their transcription and

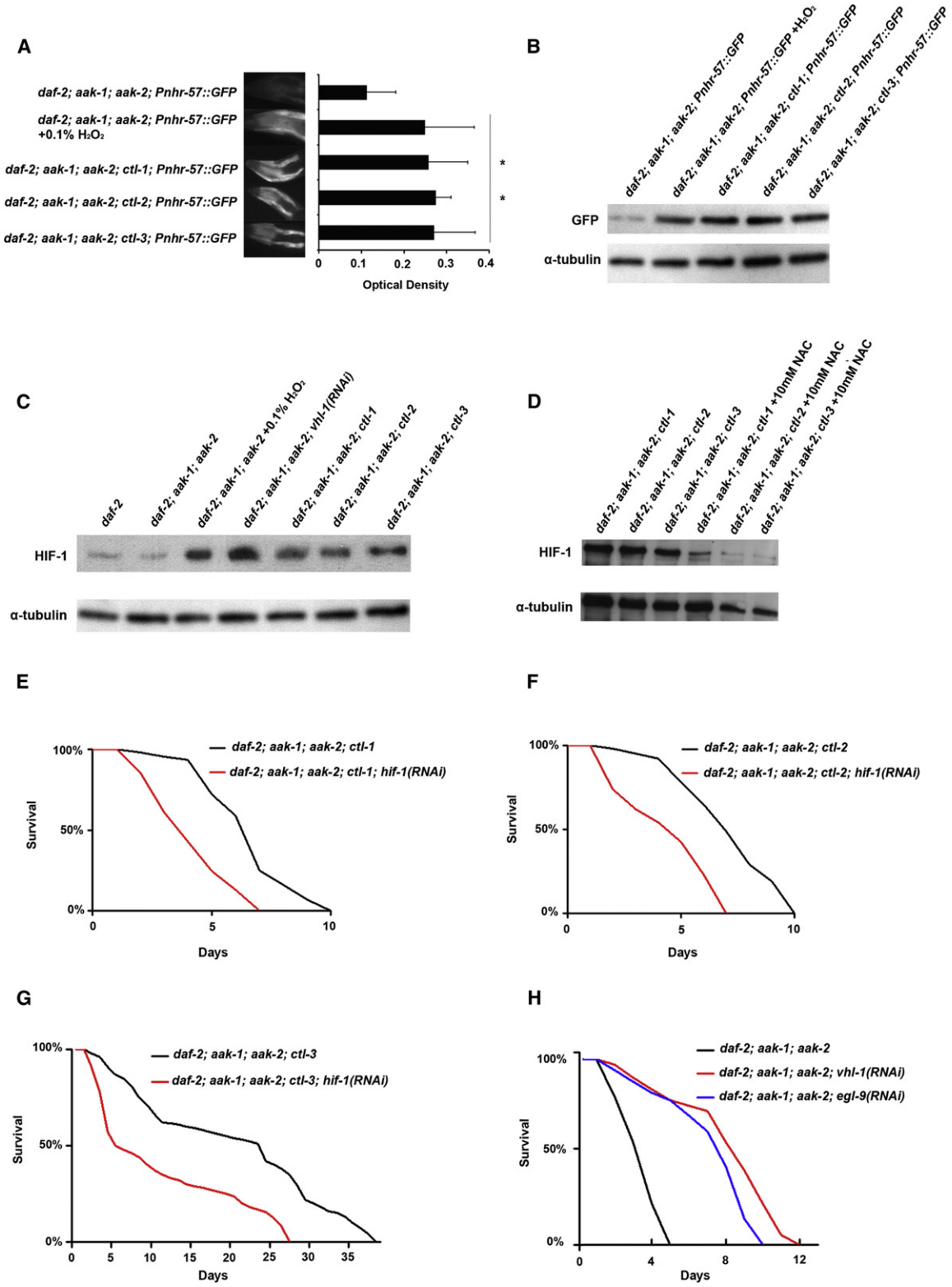

Figure 4. The Increased Survival of Catalase-Deficient AMPK Mutant Dauer Larvae Is HIF-1 Dependent

(A) AMPK mutant dauer larvae harboring *ctl* mutations or that were exposed to 0.1% H_2O_2 showed an elevated expression of a HIF-1-dependent GFP reporter. All animals were *daf-2; aak(0);* and carried the HIF-1 sensor transgene *Pnhr-57::GFP*. Error bars indicate the SD of 20 animals.

(B) Western blot analysis of GFP levels obtained from *aak(0); ctl* mutant dauer larvae or animals cultured in 0.1% H_2O_2.

(C) Western blot analysis of HIF-1 levels obtained from control and *aak(0); ctl* mutant dauer larvae or animals fed with *vhl-1(RNAi)* or cultured in 0.1% H_2O_2.

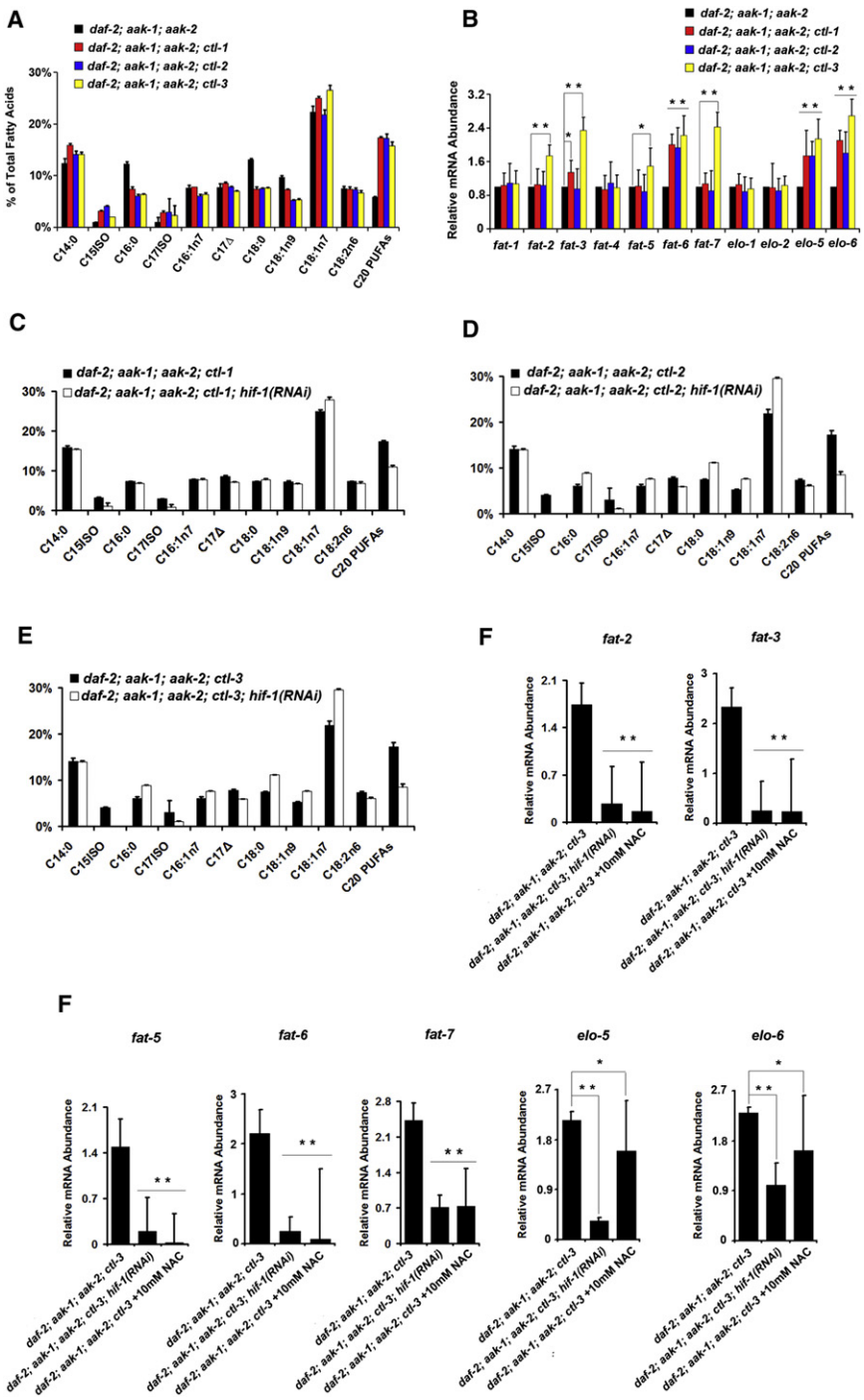

Figure 5. HIF-1 Activates Expression of Genes Involved in De Novo Fatty-Acid Synthesis

(A) *aak(0); ctl* mutant dauer larvae possess additional fatty-acid species that were not detected in AMPK mutant dauers. Total lipids were extracted from dauer larvae 24 hr after dauer formation, and the various fatty-acid species present were determined with GC/MS.

(B) The expression levels of several genes involved in de novo fatty-acid synthesis were upregulated in *aak(0); ctl* mutant dauers. Relative messenger RNA levels were analyzed with quantitative real-time PCR in dauer larvae 24 hr after dauer formation.

(C–E) The additional fatty acids detected catalase-deficient AMPK mutant dauer larvae were no longer detectable after *hif-1(RNAi)*.

(F) Both *hif-1(RNAi)* and 10 mM NAC treatment reversed the increased gene expression level of enzymes involved in de novo fatty-acid synthesis in *aak(0); ctl-3* mutant dauer larvae.

Error bars indicate the SD of three independent experiments. See also Figures S2 and S7.

hence altering the fatty-acid composition in catalase-deficient AMPK mutants, we found that all the gains in gene expression observed for each of these critical enzymes were suppressed after either *hif-1(RNAi)* or NAC treatment (Figure 5F).

Despite the fact that the dauer larva can use alternative pathways to generate macromolecular precursors (O'Riordan and Burnell, 1990; Wise et al., 2011; Metallo et al., 2012; Mullen et al., 2012), their compromise had no effect on dauer survival in the catalase-deficient AMPK mutants or the AMPK mutant dauers alone (Figure S5 and Table S2).

Taken together, our results suggest that HIF-1 activates the expression of rate-limiting enzymes that mediate desaturation and elongation during fatty-acid synthesis to stimulate the production of specific fatty-acid species, which finally contribute to the prolonged survival of the AMPK mutant dauers.

Differential Effects of Catalase Genes on Dauer Survival Reflect Varying Degrees of Oxidative Protein Damage, but Not Lipid Depletion

The removal of ATGL-1 suppresses the premature lethality of AMPK mutant dauers (Figure 6A and Table S2) by slowing the rapid hydrolysis of lipid stores, presumably providing the animals with a long-term energy source. Although we observe a similar effect on lipid stores by eliminating any one of the catalase genes, we noted considerable differences in survival

(D) Western blot analysis of HIF-1 levels in 10 mM NAC-treated *aak(0); ctl* mutant dauers.

(E–G) *hif-1(RNAi)* reversed the survival benefit conferred by each of the *ctl* mutations on AMPK-deficient dauer larvae.

(H) Both *vhl-1(RNAi)* and *egl-9(RNAi)* extended the survival of AMPK-deficient dauer larvae.

See also Figure S4 and Table S2.

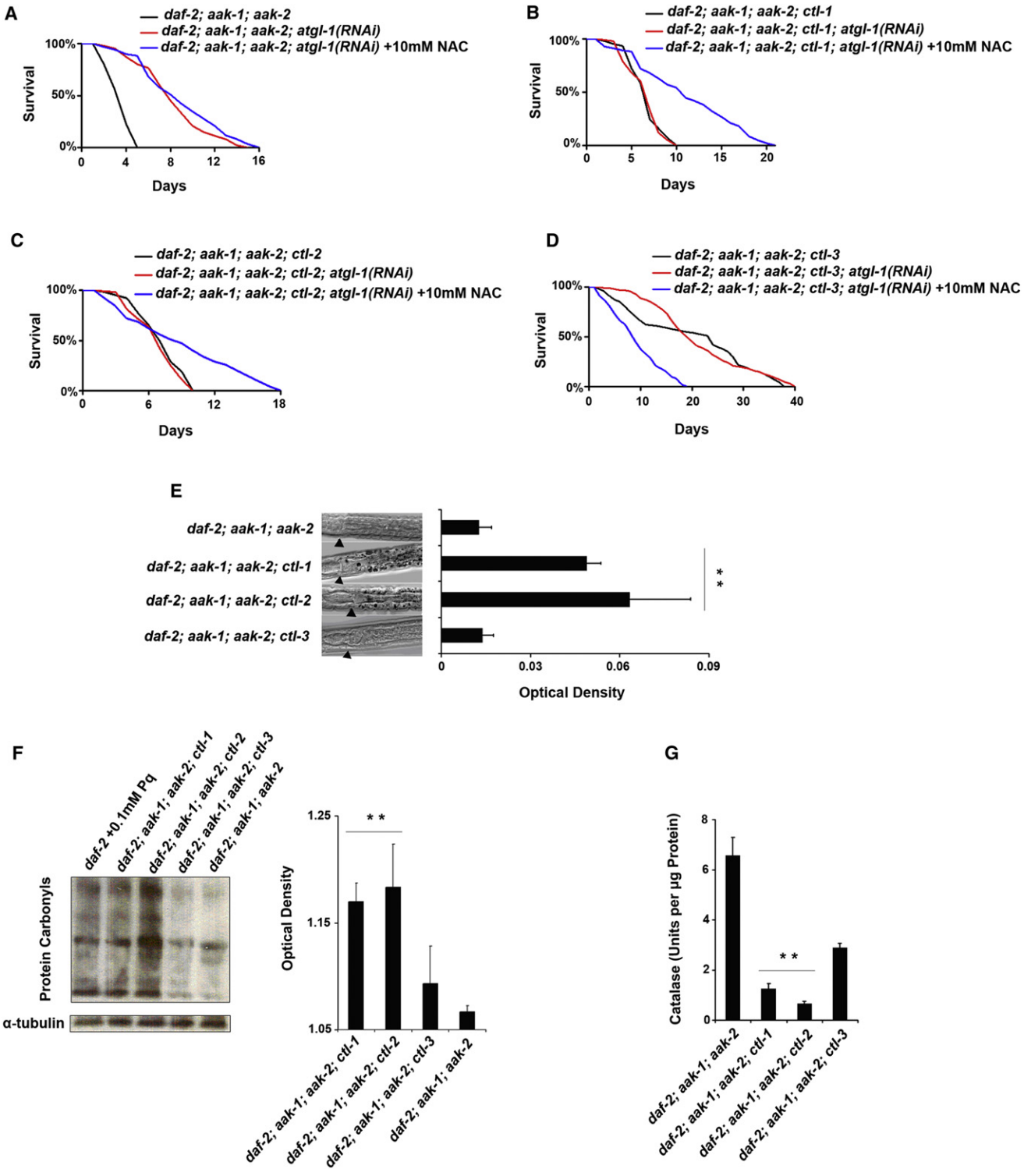

Figure 6. An Equilibrium between HIF-1-Dependent Fatty-Acid Synthesis and ROS-Induced Oxidative Damage Maximizes Survival of *ctl-3*; AMPK Mutant Dauers

(A–D) *atgl-1(RNAi)* increased the survival of AMPK mutant dauers by limiting unregulated triglyceride hydrolysis during dauer formation (A), but *atgl-1(RNAi)* had no such beneficial effect on the survival of *aak(0); ctl* mutant dauer larvae (B–D). NAC (10 mM) treatment combined with *atgl-1(RNAi)* extended the survival of *aak(0); ctl-1/2* mutant dauers (B and C) but reduced the survival of *aak(0); ctl-3* mutant dauers (D).

(E) Terminally arrested *aak(0); ctl-1 or ctl-2* larvae still stain strongly for lipids indicating that the terminal arrest of *aak(0); ctl-1* dauer larvae is unlikely to be due to premature exhaustion of lipid reserves due to misregulated *atgl-1* activity. Error bars indicate the SD of 20 animals.

between each of the mutants (Figure 1C and Table S2), while we also found that their survival could not be improved by removing ATGL-1 (Figures 6B–6D and Table S2). Furthermore, *atgl-1(RNAi)* combined with a 10 mM NAC treatment, prolonged the survival of *ctl-1/2;* AMPK mutant dauers but reversed the enhanced survival typical of *ctl-3*; AMPK mutant dauers (Figures 6B–6D and Table S2). These results led us to question whether the depletion of fat stores in these mutants was indeed the primary cause for their expiration. To address this, we determined the total fat content in terminally arrested catalase-deficient AMPK mutant to find that the arrested *ctl-1;* and *ctl-2;* AMPK mutant dauers terminate prior to the exhaustion of their available fat stores (Figure 6E), while *ctl-3;* AMPK mutant dauers were clear, indicating that their lipid stores were exhausted (Figure 6E). These observations suggested that the depletion of fat stores was not the primary reason for the terminal arrest of both the *ctl-1;* and *ctl-2;* AMPK mutant dauer larvae.

Since catalase enzymes are responsible for protecting the cell from oxidative damage and the levels of ROS were significantly increased in these mutant backgrounds, it is conceivable that the catalase-deficient AMPK mutant dauers expire because of excessive oxidative damage that would normally be buffered through the protection conferred by the catalase enzymes. ROS cause damage by introducing carbonyl groups onto proteins which can be quantified using standard immunoblotting methods. *ctl-1;* and *ctl-2;* AMPK mutant dauers demonstrated significantly higher levels of carbonylated proteins, the levels of which were comparable to control dauers treated with the strong ROS generating compound 0.1 mM paraquat (Figure 6F). On the other hand, the level of oxidative protein damage observed in *ctl-3;* AMPK mutant dauers was significantly lower than either of the two other catalase mutants, suggesting that the primary cause of terminal arrest in *ctl-1/ctl-2* AMPK mutant dauer larvae was most likely due to excessive oxidative protein/macromolecular damage, and not to depletion of lipid reserves, as was the case in AMPK and *ctl-3;* AMPK mutant dauer larvae. This was further supported by our determination of catalase activity, where AMPK and *ctl-3;* AMPK mutant dauer larvae demonstrated significantly higher catalase activity compared to the other two catalase-deficient AMPK mutant dauer larvae (Figure 6G).

Taken together, these results suggest that a balance between HIF-1-mediated fatty-acid synthesis and H_2O_2-induced oxidative damage must be achieved to maximize the survival of catalase-deficient AMPK mutant dauers. Since *ctl-3* is the least active member of the catalase family, its disruption only marginally increased the level of H_2O_2, but nonetheless to a level sufficient to trigger a substantial beneficial effect through the activation of HIF-1, yet without generating the excessive levels of oxidative protein damage typical of its paralogs *ctl-1/-2*. It is this fine equilibrium between HIF-1 activation and the damaging effects of ROS generation that explains the enhanced survival of *ctl-3* mutations compared to *ctl-1* and *ctl-2* AMPK mutant dauers (Figure 1C and Table S2).

DISCUSSION

The *C. elegans* dauer stage represents an extraordinary example of metabolic remodeling in response to environmental stress. Since dauer larvae stop feeding yet remain motile, it is imperative that the animals derive energy from intracellular sources, but without any possibility of replenishing these resources from the external environment. This is satisfied first by accumulating fat before dauer entry and later during dauer by actively maintaining pathways involved in both carbohydrate and lipid synthesis/hydrolysis throughout the duration of the diapause (Figure S2A; O'Riordan and Burnell, 1990; Perez and Van Gilst, 2008).

The regulation of these pathways in *C. elegans*, like in most organisms, is largely dependent on AMPK, a master regulator of metabolic homeostasis. In the dauer larva, AMPK attenuates the activity of the triglyceride lipase ATGL-1 to protect long-term energy stores (Narbonne and Roy, 2009). In situations of compromised AMPK signaling, ATGL-1 activity is abnormally high, reducing the global triglyceride levels remarkably, consequently leading to premature dauer lethality. Curiously, some fatty-acid species are substantially reduced in AMPK mutant dauers (Figure S2B), suggesting that AMPK not only regulates triglyceride hydrolysis in dauer larvae, but must also impinge on fatty-acid biosynthesis, perhaps through its well-known targets *ACC1/ACC2* (*pod-2* in *C. elegans*). Whether these fatty-acid species are required to contribute to the overall lipid/energy pool or whether they may play additional roles in signaling during the dauer stage still remains to be determined. Recent data have shown that alterations in the AMPK-dependent regulation of fatty-acid synthesis can have widespread positive and negative implications for cellular homeostasis, suggesting that AMPK compromise may extend beyond the maintenance of the lipid pool during dauer (Jeon et al., 2012). Nevertheless, many of the metabolic changes typical of dauer development most likely involve AMPK. In the absence of AMPK, dauer larvae that are compromised for insulin signaling are incapable of readjusting to the physiological constraints imposed by this state and as a result expire prematurely.

Using a nonbiased genome-wide survey, we identified 551 RNAi clones that affect the survival of prematurely arresting AMPK mutant dauers. These gene identities will help us to build a framework to better understand how AMPK converges on a plethora of cellular processes required to promote the typical long-term survival of the dauer larva. Among the most common groups of genes we identified in our screen were nuclear

(F) Loss of catalase gene function results in a substantial increase in oxidative damage. *aak(0); ctl-3* mutant 1-day-old dauer larvae have comparatively less oxidative damage at the protein level, as determined by OxyBlot detection, than do *aak(0); ctl-1 and aak(0); ctl-2* 24 hr after their switch from dauer larvae. Band intensity was quantified by measurement of the optical density followed by subsequent processing with Openlab software. Error bars indicate the SD of three independent experiments.

(G) Catalase activity was significantly lower in both *aak(0)* and *aak(0); ctl-3* mutant dauer larvae compared to *aak(0); ctl-1* or *aak(0); ctl-2* mutant dauer larvae. Error bars indicate the SD of three independent experiments.

See also Table S2.

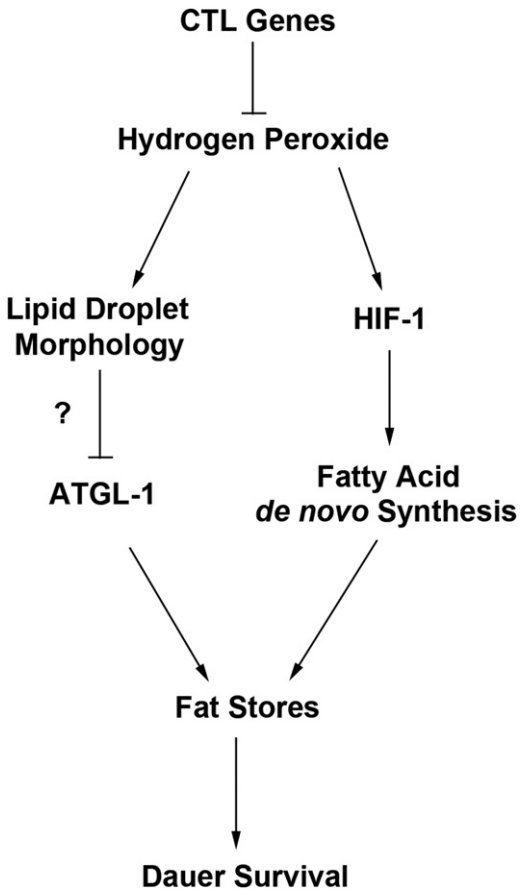

Figure 7. H_2O_2-Mediated Hormesis Prolongs Survival in AMPK Mutant Dauer Larvae via HIF-1-Dependent and -Independent Effects on Lipid Metabolism

Elevation of global H_2O_2 due to loss of any member of the CTL family has two beneficial effects on AMPK mutant dauer survival—expansion of lipid droplet size, which may attenuate the activity of ATGL-1, and activation of HIF-1 to promote fatty-acid biosynthesis—both of which act to restore fat stores for prolonged dauer survival.

hormone receptors, ubiquitin ligases, F-box-containing proteins, and Zinc finger-containing proteins, setting the stage for further interrogation of their individual roles in the typical metabolic remodeling that accompanies dauer development.

Our data also suggest that the peroxisome may play a central role in regulating dauer survival since peroxide-consuming enzymes, such as *mlt-7*, T06D8.10, and *prdx-3*, and a gene that affects peroxisomal fatty-acid oxidation encoded by ZK550.5, a peroxisomal phytanoyl-CoA hydroxylase enzyme, were also identified in our RNAi screen (Table S1). Other genes that affect mitochondrial function or genes such as malate dehydrogenase (*mdh-1*), which generates NADPH to reduce peroxide levels, prolong dauer survival in the AMPK mutants (Figure S6 and Table S2), although some of these were not identified in our screen due to our cutoff criteria.

Not all gene products that alter peroxisome function or morphology are involved in this pathway: several peroxisomal genes that have been characterized for their roles in the maintenance of lipid droplet morphology were not identified as suppressors of the AMPK-dependent lethality in dauers (Zhang et al., 2010). The candidates that we identified most likely affect cellular H_2O_2 levels, and it is the compromise of this function of the peroxisome that enhances the survival of AMPK mutant dauer larvae.

Because we identified both *ctl-1* and *ctl-2* in this screen, we focused on how the loss of catalase gene function could confer organismal survival in a situation where energy resources are drastically limited, namely during the dauer diapause in *C. elegans*. The observed beneficial effect of each of the individual catalase genes on AMPK mutant dauer survival is somewhat curious based on their described expression patterns (Petriv and Rachubinski, 2004). However, each of the catalase genes is significantly upregulated in essentially all tissues in dauer larvae (Figure S1), suggesting that a threshold level of organismal catalase activity is required to counteract the general increase in ROS typical of dauer (Figure 3A). Nonetheless, in addition to their effects on premature dauer lethality, the individual compromise of these genes also blocked the abnormally rapid hydrolysis of fat stores, while also improving the osmosensitivity of AMPK mutant dauers, essentially correcting all of the AMPK-dependent metabolic defects that we have characterized in the dauer larva.

More importantly, however, the loss of these gene functions allowed us to unveil a previously uncharacterized role of H_2O_2 as a major effector of lipid homeostasis during nutrient stress. In this sensitized background where H_2O_2 is allowed to accumulate, we were able to demonstrate how its increased levels can readjust the fatty-acid biosynthetic machinery to ultimately prolong the survival of the dauer larvae, despite the metabolic challenges it must confront due to the loss of AMPK function. Curiously, the increases in H_2O_2 rescue all the metabolic defects that arise due to the disruption of AMPK by activating HIF-1 to alter gene expression to favor fatty-acid biosynthesis, but also through its HIF-1-independent effects on ATGL-1 activity, the molecular basis of which is still unclear (Figure 2B).

Our data strongly indicate that incremental changes in H_2O_2, a major contributor to intracellular ROS levels, can extend dauer survival when suprathreshold levels activate a sensitive intracellular oxygen sensor, HIF-1. Many physiological conditions have been shown to affect HIF-1 activity and most of them do so by protecting HIF-1 from VHL-mediated degradation. Previous findings have implicated H_2O_2 in perturbing PHD function through its ability to drive the Fenton reaction to decrease the effective cellular concentrations of Fe II (Gerald et al., 2004). Whether the observed increases in H_2O_2 affect the cellular Fe II oxidation state in AMPK mutant dauers is unclear, but such changes may provide a plausible explanation for the HIF-1 accumulation. By blocking PHD function, HIF-1 accumulates, allowing this critical factor to readjust the entire transcriptional repertoire of the organism to better adapt to the perceived environmental stress and prolong survival in the stressed state (Figure 7).

Over the course of the last century, ROS have been implicated in several different contexts, where they almost invariably have been shown to interrupt cellular functions by damaging macromolecules involved in key cellular/physiological processes. These negative effects of ROS have recently been critically evaluated using genetically tractable systems (reviewed by Hekimi et al., 2011; Owusu-Ansah and Banerjee, 2009). The outcome of this body of work is perplexing since, quite unexpectedly,

low to intermediate concentrations of ROS were actually found to be beneficial for some of these processes, namely stem cell function and life-span extension.

So how could such a vilified family of molecular intermediates act in a beneficial way? In 1943, Southam and Ehrlich described how subcritical concentrations of toxic compounds could confer cellular benefit through a presumptive intracellular adaptation mechanism that was referred to as "hormesis." This phenomenon has been demonstrated for many toxic compounds (Cook and Calabrese, 2006; Heinz et al., 2010), and, recently, this same effect has been shown for ROS in *C. elegans* (Schulz et al., 2007; Mouchiroud et al., 2011; Lee et al., 2010).

The prosurvival benefits we describe here are entirely attributable to increases in H_2O_2 levels within the animal. These beneficial effects correspond to a limited range of H_2O_2 levels that likely approaches, but does not exceed some threshold concentration, above which results in macromolecular damage. This is best demonstrated in *ctl-1* and *ctl-2* mutants, in which any benefit that these mutations confer on dauer survival through the generation of extra lipid/energy is counterbalanced by the extensive oxidative protein damage that eventually results in terminal arrest. In contrast, loss of *ctl-3* gene increases the levels of H_2O_2 only modestly, placing it in the optimal range to activate a HIF-1-dependent transcriptional effect without the consequences of ROS-mediated macromolecular damage. Our data therefore provide a molecular basis to account for the hormesis attributed to cases of low to intermediate levels of H_2O_2 and/or ROS production.

The activation of a HIF-1-dependent transcriptional program that affects cellular energy stores is indeed critical for adapting to energy stress and prolonging the survival of these dauer larvae. These changes are typical of the metabolic adjustments that occur in cancer cells associated with the Warburg effect, where energetically sound pathways are modified to provide a means of satisfying the unique growth requirements of these rogue cells, while consequently compromising their energetic efficiency (Deberardinis et al., 2008).

In this light, the events that occur during the nutrient-constrained dauer stage may be representative of what occurs during tumorigenesis. The tumor environment can be very dense, and therefore the internal regions are often nutrient-depleted and largely hypoxic. This favors HIF-1 activation, which drives the transcription of numerous factors that will ultimately impinge on many angiogenic and metabolic regulators (Semenza, 2010). This is very common in rapidly growing, aggressive tumors and is often associated with poor prognosis (reviewed by Dewhirst, 2009). Our data indicate that in addition to the known effects of HIF-dependent transcription on angiogenesis and cell growth, it may also dramatically alter the expression of several rate-limiting enzymes required for lipid biosynthesis. These changes in lipid synthesis may in turn directly or indirectly affect other processes that impinge on cell survival (Jeon et al., 2012).

If HIF-1 can affect the ability of the tumor cells to generate their own energy by modifying their capacity for lipid biosynthesis, its activation in the cells deep within the tumor would make them less dependent on nutrient delivery, while also releasing them from external regulatory constraints, thereby enhancing their potential for unscheduled growth. Because AMPK would normally play a protective role to block cell growth in response to poor nutrient availability or during oxidative stress, it would seem imperative to evaluate whether activation of HIF-1 is associated with the loss of LKB1 or AMPK in various tumors, or in patients with tumor predisposing syndromes such as Peutz Jeghers Disease.

EXPERIMENTAL PROCEDURES

Feeding RNAi

Our feeding RNAi protocol was performed as described (Kamath et al., 2001). In brief, L3–L4 stage hermaphrodites were transferred onto regular plates seeded with individual double-stranded RNA-expressing bacterial clones. The animals were transferred to a new set of seeded plates after 1 day of incubation at 15°C, and phenotypes were scored for the F2 generation.

Dauer Survival

Dauer survival was determined as described elsewhere (Narbonne and Roy, 2006). In brief, dauer larvae were kept in double-distilled water or with dissolved chemicals. Survival was scored according to their appearance and moving response to a gentle tap on the plate. For H_2O_2 treatment, treated animals were protected from light with aluminum foil and were exposed to freshly prepared H_2O_2 solution daily to prevent H_2O_2 decomposition.

Oil Red O Staining of Dauer Larvae

Oil red O staining of dauer larvae was performed as described (Soukas et al., 2009). Dauer larvae were fixed in 2% paraformaldehyde and stained with 60% oil red O solution. Stained dauer larvae were observed and imaged with a Zeiss Imager.21 microscope equipped with a Hamamatsu camera and differential interference contrast (DIC) optics. Optical density was determined with OpenLab software (Improvision).

Worm Extract Preparation

Worm extracts were prepared by sonication and subsequent centrifugation. Protein concentration was determined with a NanoDrop 2000c spectrophotometer (Thermo Scientific).

Triglyceride Quantification

Triglyceride content was determined with a commercially available kit (Sigma-Aldrich) according to the manufacturer's recommendations. Absorbance was measured with a NanoDrop 2000c spectrophotometer at 540 nm. All calculated triglyceride concentrations were finally normalized to protein concentration.

Osmotic Resistance Assay

Osmotic resistance of dauer larvae was performed as described (Narbonne and Roy, 2009). The survival of day 4 dauer larvae was scored after being exposed to solutions of varying NaCl concentrations for 24 hr at 25°C.

Quantification of Lipase Activity

Lipase activity for dauer animals was measured as described (Narbonne and Roy, 2009) with a commercially available QuantiChrom kit from BioAssay Systems according to the manufacturer's recommendations. Optical density values were measured with a Varioskan Flash Multimode Reader version 3.00.7 at the wavelength of 412 nm.

C1-BODIPY-C12 Staining

C1-BODIPY-C12 staining was performed as described (Mak et al., 2006). Synchronized L1 larvae were transferred to regular plates with C1-BODIPY-C12 and grown at 25°C until they reach the dauer stage. C1-BODIPY-C12 stained dauer larvae were observed and imaged with a Zeiss Imager.21 microscope outfitted with DIC optics equipped with a Hamamatsu camera. Lipid droplet diameter was measured with Openlab software, and volume was calculated with the following formula: $4/3 \times \pi \times (\text{diameter}/2)^3$.

Measurement of Reactive Oxygen Species

ROS levels were quantified as described elsewhere (Lee et al., 2010). Worm extracts were incubated with 2′,7′-Dichlorofluorescein Diacetate (DCF-DA).

Fluorescence intensity was measured with a Varioskan Flash Multimode Reader version 3.00.7 at the excitation wavelength of 485 nm and the emission wavelength of 535 nm.

Hydrogen Peroxide Quantification

Hydrogen peroxide level was determined with a commercially available Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) according to the manufacturer's recommendations. The fluorescence was measured with a Varioskan Flash Multimode Reader version 3.00.7 at the excitation wavelength of 540 nm and the emission wavelength of 560 nm. The fluorescence intensity was normalized by subtracting the background fluorescence of the Amplex Red reagent/HRP working solution.

Gas Chromatography/Mass Spectrometry Analysis

Fatty acids were extracted into the organic phase as described (Miquel and Browse, 1992). Gas chromatography was performed on a HP6890N instrument (Agilent) equipped with a DB-23 column (30 m × 250 μm × 0.25 μm). The column was run at a constant flow mode at 0.8 ml/min. The initial oven temperature was 160°C with an initial time of 1 min. It was increased to a final temperature of 240°C at a rate of 4°C/min with a final time of 10 min. The detector was at 240°C. Fatty-acid species were identified by comparison with fatty-acid standards (C8-C24, Supelco) and mass spectrometric analysis.

RNA Isolation and Real Time PCR

Total RNA was extracted with Trizol (Invitrogen) and purified with the RNeasy kit (QIAGEN) according to the manufacturer's recommendations. RNA concentration and purity were determined with a NanoDrop 2000c spectrophotometer. Purified RNA (0.5 μg) was used to synthesize complementary DNA. Gene expression levels were determined by real time PCR with the SYBR Green Supermix and BioRad iCycler Real Time PCRSystem (BioRad). Relative gene expression was normalized to *act-1* and *cdc-42* as internal loading control.

Oxidative Protein Damage Level Measurement

The level/degree of oxidative protein damage was measured with a commercially available kit (Oxyblot) as described (Yang et al., 2007). Band intensities were measured with OpenLab software and normalized to α-tubulin present in the same sample.

Statistical Analysis

Values are shown as mean + SD. Comparison of mean values was evaluated by one-way ANOVA followed by a Tukey HSD test. A p value less than 0.05 was considered significant. In all experiments, the number of asterisks represents the following: * $p < 0.05$ and ** $p < 0.01$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.07.016.

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